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14. ABSTRACT Our studies evaluate how recurrent dehydration can cause chronic kidney disease, an important question for the military and public. We hypothesize, based on preliminary data, that dehydration associated renal injury results from hyperosmolarity induced activation of renal aldose reductase-fructokinase. We made excellent progress this last year. First, we now have the floxed KHK KO mouse and have generated successful litters so we can proceed with selectiverenal knockout of fructokinase in dehydration induced kidney disease. Aim 2 investigates the role of vasopressin receptors and uric acid, and we have completed studies with the Vasopressin 2 receptor (submitted) and have completed the experiments with V1a and V1b knockout. We have also performed some hypothalamic explant studies and documented a functional fructokinase system in the hypothalamus (manuscript submitted). Aim 3 tests the role of rehydration with fructose solutions with or without blocking of vasopressin receptors. The administrative delay with our collaborators has been resolved and experiments are ongoing. In summary, we are proceeding with how recurrent dehydration causes chronic kidney disease via vasopressin and fructokinase and remain on target for finishing in time.

15. SUBJECT TERMS
Dehydration, Chronic kidney disease, vasopressin, fructose

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1. INTRODUCTION:

Our studies are aimed at identifying how recurrent dehydration may lead to chronic kidney disease. This is important for the military as well as for the general population. In preliminary studies we developed the first model of chronic kidney disease from recurrent dehydration and found evidence that the renal injury is mediated by hyperosmolarity that activates an enzyme pathway in the kidney (aldose reductase-fructokinase) that leads to tubular injury. Here we will proceed with three aims to further identify the role of this pathway. Aim 1 will evaluate the role of selective knockout of fructokinase in the renal tubule on dehydration induced kidney disease. Aim 2 will investigate the role of downstream mediators, including the role of vasopressin receptors and uric acid in driving dehydration induced kidney injury. Aim 3 will evaluate the role of rehydration with fructose solutions with or without blocking of vasopressin receptors. These studies should provide major insights into how recurrent dehydration may cause chronic kidney disease

2. KEYWORDS: *Dehydration, Chronic kidney disease, vasopressin, uric acid, fructose*

3. ACCOMPLISHMENTS:

What were the major goals of the project? According to our revised and accepted SOW, our primary goals consisted of three aims:

Aim 1 involved generating an inducible tissue specific knockout mice for fructokinase. This required generating a mouse whose fructokinase gene is floxed (the floxed fructokinase mouse) and then selectively knocking fructokinase from the renal tubule. During the first year we generated the KHK floxed mouse, but unfortunately the original mouse did not establish any progeny with the floxed site. However, this last year we rederived the floxed fructokinase knockout mouse, and to our delight we have now generated litters of floxed mice. We are now set to cross this mouse with a Cre mouse targeting the tubule, and we should be on target to complete this study in the next year.

Aim 2 involves testing the role of vasopressin and uric acid in our mouse model of recurrent dehydration induced chronic kidney disease. There are 4 sets of experiments—studies using V1a and V1b receptor knockout mice, studies using wild type mice administered allopurinol to lower uric acid, and a study using desmopressin that only stimulates vasopressin type 2 receptors. We have completed three of these studies (V1a, V1b, and V2 experiments) and the allopurinol study will be performed this fall. The desmopressin (V2 agonist) showed marked worsening of the disease and the paper is under review at Am J Physiol. The V1a/V1b data is currently being analyzed, but the preliminary data suggests the V1b may be more susceptible to injury whereas the V1a may not.

We also completed an analysis of the aldose reductase-fructokinase axis in the hypothalamus in the setting of acute dehydration, including the use of hypothalamic explants (an additional experiment in Aim 2) and documented not only a role for endogenous fructose in mediating vasopressin synthesis and release, but also for a key role for hypothalamic fructokinase in driving these effects. This revised paper will be submitted to the J Neurophys (revised). In terms of milestones, we are well on target to complete our studies in a timely way.

Aim 3 tests the effect of rehydration in our model of chronic kidney disease, by comparing the administration of fructose versus water and the impact of blocking vasopressin in this process. The previous administrative challenges in executing the subcontract to Instituto Nacional de Cardiología Ignacio Chavez have been resolved, and experiments are underway. Our collaborator, L. Gabriela Sanchez-Lozada, is initiating the studies of dehydration associated renal damage by determining the effect of tolvaptan to reduce injury by blocking V2 receptor pathways. These studies, coupled with the planned renal hemodynamic studies, are on target for completion this coming year.

Thus, we remain on track for completion of all aims, and to meet all of our milestones prior to completing the experiments next year.

What was accomplished under these goals?

1) Major activities.

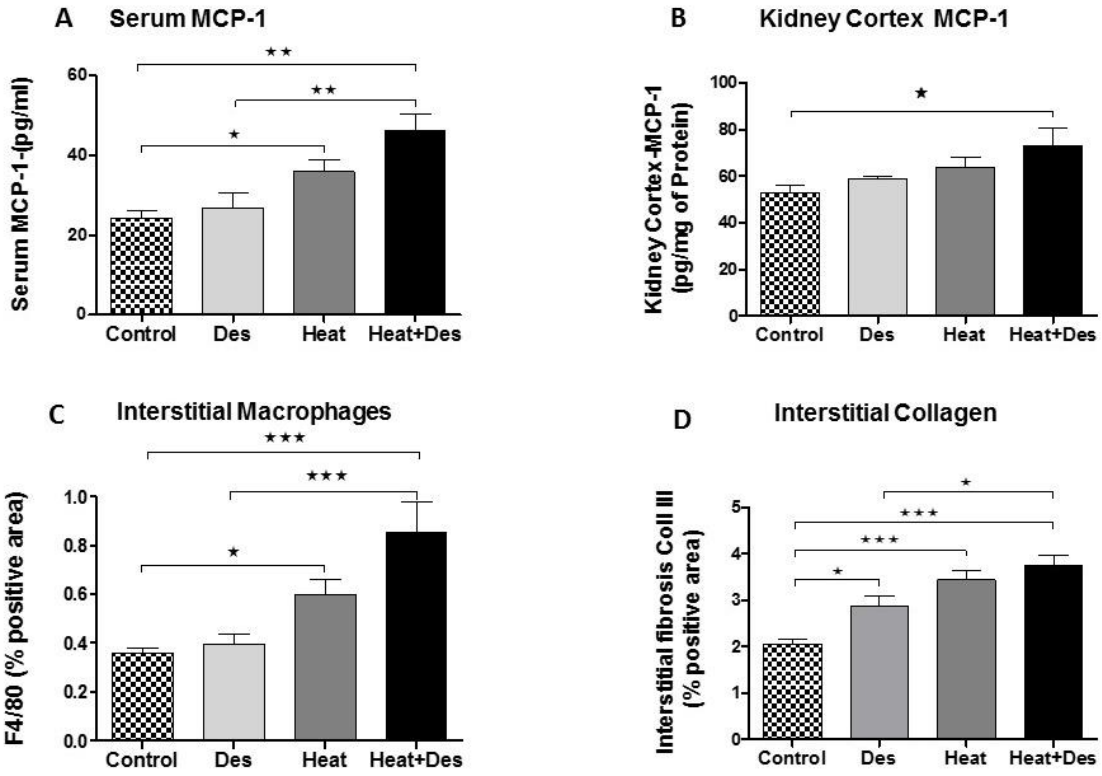
The primary activities included a) Generation of the Floxed Fructokinase knockout mouse; b) Breeding the Vasopressin 1a and 1b receptor knockout mouse; c) Demonstration that stimulation of vasopressin 2 receptors with desmopressin worsens dehydration-associated kidney disease. d) Documenting that endogenous fructose is generated with acute dehydration and that it mediates vasopressin synthesis in the brain through a fructokinase-dependent mechanism, and e) completion of the studies on dehydration induced renal injury using the V1a and V1b knockout mice. These studies are discussed in detail below.

2) Specific objectives;

a. The goal of Aim 1 for the first year was the development and breeding of a loxP mouse that would allow us to test the role of rena-specific fructokinase in mediating dehydration associated chronic kidney disease. During the first year we generated a loxP fructokinase mouse but unfortunately it was not successful at breeding. This last year we rederived the loxP fructokinase mouse, and we have successfully generated founders who have had successful litters. We plan to cross thiw mouse with the TetOn LC-1 a Pax8-rTA Cre transgenic mouse. This will allow us to determine the role of fructokinase in the kidney in our model of heat-dehydration induced renal injury

(aim 1) but will also be invaluable for future studies on the role of fructokinase in other organs (liver, brain, adipose, islets).

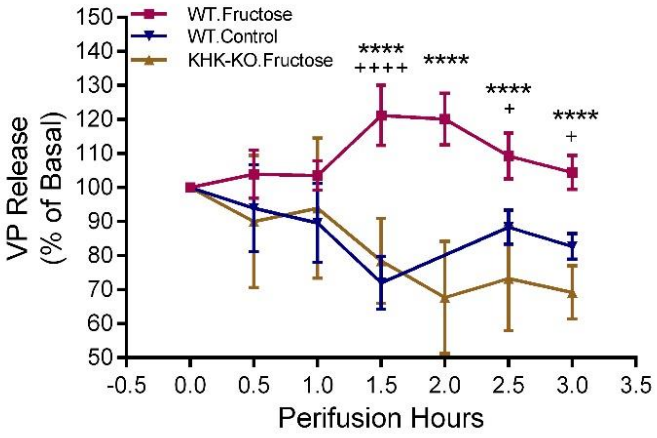
b). The goal of Aim 2 was to evaluate the role of vasopressin receptor 1A, vasopressin receptor 1B, vasopressin 2 receptor, and uric acid in the model of heat-dehydration induced kidney disease. The first experiment we finished was the desmopressin (V2 agonist) study, in which we found that the V2 agonist markedly worsened dehydration induced renal injury. The study showed an impressive effect of desmopressin on worsening tubulointerstitial injury, but desmopressin also induced glomerular changes. The paper is extensive and is currently under review at American Journal of Physiology Renal Physiology. We enclose a figure below showing the stepwise worsening of renal injury when desmopressin is administered.



We also have completed our studies using the V1a and V1b knockout mice. One of the interesting findings with the V1b knockout was that it was quite sensitive to heat, and we found we had to reduce the severity of the heat exposure to minimize morbidity and mortality. The tissues are now undergoing analysis, and the allopurinol study will be initiated in October.

In addition, another component of Aim 2 was to dissect out the fructose-vasopressin axis during acute dehydration and to include hypothalamic explant studies to look at direct control of hypothalamic function as it related to the role of fructose in stimulating vasopressin. I am delighted to state that we have completed this study and confirmed that fructokinase is present in the hypothalamus by PCR and in situ hybridization, and we have shown in the explants that fructose can stimulate vasopressin synthesis in wild type explants but not hypothalamic

explants from fructokinase knockout mice. The revised paper is under review at the Journal of Neurophysiology, and one of the key figures is shown below.



3) Significant results/key outcomes

Our primary successes include 1) the development of a Lox-P fructokinase mouse with successful generation of a Lox-P colony that will allow completion of our proposed Aim 1; 2) Completion of the DDAVP study with the manuscript under review at AJP Renal Phys, and the completion of the acute dehydration and explant study with the manuscript under review at the J of Neurophysiology. We have also completed the studies investigating the effect of knocking out V1 and V1b on dehydration-induced renal injury and the tissues are currently under analysis. The third aim is also underway with studies to determine if tolvaptan will block renal injury. The primary significance of the current studies is we have identified, for the first time, of a novel mechanism driving vasopressin release, and that it by a hyperosmolarity-driven upregulation of aldose reductase with the generation of endogenous fructose leading to a fructokinase dependent vasopressin synthesis. Thus, we have identified a whole new system driving vasopressin release. Second, we have shown that too much vasopressin is bad for the kidney, and that it can induce renal damage from dehydration, particularly through the V2 receptor pathway. Finally, we have initiated the converse, which is to see if we can protect dehydration induced kidney damage by blocking the V2 receptor.

4) Other achievements.

The development of the Floxed fructokinase mouse will have major implications for future studies that extend well beyond the DOD grant. First, it will allow us to explore the role of CNS fructokinase in sugar craving, and of islet cell fructokinase in the development of diabetes. The liver fructokinase can also be targeted and may be

responsible for the fatty liver and insulin resistance. Of course, targeting the kidney might provide the insight of how dehydration causes kidney injury via the fructokinase pathway.

In addition, this last year we have determined that the epidemic of kidney disease due to dehydration and heat stress is present in multiple countries and, working with climatologists, we linked it to climate change. The paper was published in CJASN and led to numerous interviews on NPR and elsewhere. We also reviewed the evidence vasopressin and fructokinase are involved in the mechanisms of dehydration, and this paper was published in JASN. The identification of these ongoing epidemics, coupled with the research in this grant identifying potential mechanisms by which kidney damage may be occurring, should be of great benefit in the development of clinical trials to prevent chronic kidney disease that is occurring from recurrent dehydration.

5) Challenges/ stated goals not met.

During the first year of our grant there were administrative challenges related to getting approval from the IACUC and ACCURO for the dehydration studies in our animals, but all was eventually approved. There were also some challenges with getting our subcontract with Mexico approved by the two Universities—however, this was also resolved and the studies in Mexico are now ongoing.

What opportunities for training and professional development has the project provided?

The grant was not meant to provide training or professional development, and so at one level there is nothing to report. However, the studies are opening up information on the role of dehydration in kidney disease. This last year our work was selected to be the primary topic for a meeting in Aspen organized by the Aspen Global Change Institute, and our work has also led to invited presentations at the American Society of Nephrology in Chicago in November 2016 and at the European Dialysis and Transplant meeting in Madrid in June 2017. We were also invited to speak at the American Public Health Association Conference in Denver in September 2016. I have also been asked to speak in several graduate school courses, including a T32 funded course at the University of Colorado on our work to help in the education of physicians and graduate students interested in the effects of heat stress on health.

How were the results disseminated to communities of interest?

There have been multiple news reports on our work, including in NPR and various journals. The discovery of multiple epidemics of chronic kidney disease among workers in Central America, Mexico, India and Sri Lanka has been international news, and the strongest evidence to date is that it is mediated by heat stress and dehydration. Our work linking it with vasopressin and fructose metabolism has often been quoted. Hopefully as we identify the specific mechanisms, new therapies can be tested that might have an impact on disease.

What do you plan to do during the next reporting period to accomplish the goals?

We are poised to complete all Aims in the next year. The Cre-floxed mouse studies from Aim 1 should be performed in the spring of 2017, and the allopurinol studies in Aim 2 should be done this fall. Aim 3 is currently in process, as is the analysis of the V1a and V1b knockout mice in Aim 2. Two papers have already been submitted. Thus, we are delighted that our work remains on target with our projected milestones.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our studies document for the first time the importance of chronic recurrent dehydration as a mechanism for causing chronic kidney disease. This has led to huge interest from the academic nephrology societies and public media around the world, especially as it is becoming apparent that there are epidemics of chronic kidney disease emerging in Central America, India and Bangladesh, Sri Lanka, and elsewhere. As such, there is great interest in our research, especially with the realization that this disease may increase with global warming and worldwide water shortages. Our DOD studies are elucidating the role of the vasopressin and fructokinase systems, and also the importance of the rehydration fluid. This year we have made the floxed fructokinase knockout, completed the vasopressin receptor knockout mice studies, performed the hypothalamic explant studies and documented the presence of the fructokinase-vasopressin axis in the hypothalamus, and initiated studies evaluating the effect of fructose rehydation and the role of the V2 receptors from Aim 3. We have already shown that it is the vasopressin receptor 2 that is responsible for much of the renal injury induced by dehydration. As the analyses are ongoing, it is evident that the impact of the DOD studies is quite significant, as it is elucidating mechanisms of kidney damage from recurrent dehydration. With the recent reports that there have been over 40,000 deaths from these epidemics worldwide, the importance of our work is extremely high.

What was the impact on other disciplines?

Our work is identifying global warming as a factor driving kidney disease, and as such is causing some concern as the first epidemic disease induced by climate change. This has generated interest in many other disciplines (general medicine) as well as by the lay public (NPR, BBC). The Aspen Global Change Institute recently invited our group as well as climatologists, anthropologists, epidemiologists, and interested physicians to a week-long conference on climate change and health in which our group was the spotlight. The DOD grant is exploring the mechanisms, and as the results are generated, will likely have an impact on our understanding of

the cause of disease and the importance and risks associated with dehydration. We believe our reports will identify chronic kidney disease as the first major human disease due to global warming.

What was the impact on technology transfer?

Our work is heightening interest into the correct ways for rehydration when in the dehydrated state. Indeed, our work is generating concern that the current use of WHO sugar rehydration packages for dehydration may have injurious effects on kidney health (based on preliminary studies performed by Dr Sanchez-Lozada in preparation for specific aim 3 studies of the DOD proposal). While this data is not generating new intellectual property, it is generating interest in current approaches to the treatment of dehydration.

What was the impact on society beyond science and technology?

Our studies could lead to a reevaluation of sports drinks and rehydration packages for the hydration of individuals who are exposed to heat and dehydration.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No significant changes were made to the approach other than a minor revision of the Statement of Work plan as it related to Aim 3. The proposed change to the SOW was submitted and approved by the DOD in the summer of 2015.

Actual or anticipated problems or delays and actions or plans to resolve them

There were two delays in the first year of the study—the first was obtaining animal care protocol by both our local IACUC and ACCURO. This however, was approved in mid-2015. The second delay related to obtaining an agreement between my institution (University of Colorado) and our collaborator's institution (Cardiologia University in Mexico City) as it related to the subcontract. This also was resolved in early 2016. Currently, there are no anticipated problems or delays.

Changes that had a significant impact on expenditures

There were no changes that had impact on expenditures this last year

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

Publications, conference papers, and presentations

Nothing to report (yet)

Books and book chapters

Nothing to report

Other publications, conference papers, and presentations.

1. Glaser J, Lemery J Rajagapolan B, Diaz HF, Garcia-Trabanino R, Taduri G, Madero M, Amarasinghe M, Abraham G, Anutrakulchai S, Jha V, Stenvinkel P, Roncal-Jimenez C, Lanaspa MA, Correa-Rotter R, Sheikh-Hamad D, Burdmann EA, Andres Hernando A, Milagres T, Weiss I, Kanbay M, Wesseling C, Sanchez-Lozada LG, Johnson RJ. Climate Change and the Emergent Epidemic of Chronic Kidney Disease from Heat Stress in Rural Communities: The Case for Heat Stress Nephropathy. CJASN 2016 Aug 8;11(8):1472-83
2. Johnson RJ, Stenvinkel P, Jensen T, Lanaspa MA, Roncal C, Song Z, Bankir L, Sanchez-Lozada LG. Metabolic and Kidney Diseases in the Setting of Climate Change, Water Shortage, and Survival Factors. JASN 2016 Aug;27(8):2247-56
3. Roncal-Jimenez CA, Milagres T, Andres-Hernando A, Kuwabara M, Jensen T,Song Z, Bjornstad P, Garcia GE, Sato Y, Sanchez-Lozada LG, Lanaspa MA and Johnson RJ. Desmopressin (Type 2) Vasopressin Receptor Agonist Accelerates Heat Stress Nephropathy in Mice. Submitted to AJP Renal
4. Song Z, Roncal-Jimenez CA, Lanaspa-Garcia M, Oppelt SA, Kuwabara M, Jensen T, Milagres T, Andres-Hernando A, Ishimoto T, Garcia GE, Johnson G, MacLean PS, Sanchez-Lozada LG, Tolan DR, Johnson RJ. Role of Fructose and Fructokinase in Acute Dehydration Induced Vasopressin Gene Expression and Secretion in Mice. Submitted to J Neurophysiol

Website(s) or other Internet site(s)

We do not have a website that details our results of the DOD study

Technologies or techniques

We have not introduced any new technologies or techniques.

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Our research is generating great interest in the role of dehydration and global warming in chronic kidney disease. Specifically, by identifying vasopressin and fructokinase as potential targets, it may stimulate interest in the use of inhibitors of these substances as potential therapies to prevent renal injury from heat stress.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	*Richard J Johnson*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	2 months
Nearest person month worked:	
Contribution to Project:	*Dr. Johnson has overseen the design, performance and analysis of the studies*
Funding Support:	*DOD funding*

Name:	*Zhilin Song*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 months
Contribution to Project:	*Dr. Song is performing studies to identify the effects of dehydration on the vasopressin axis in the hypothalamus.*
Funding Support:	*DOD funding*

Name:	*Carlos Roncal*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 months
Contribution to Project:	*Mr. Roncal is performing all of the experiments in Aim 2 and overseeing their analyses.*
Funding Support:	*DOD funding*

Name:	*Laura G Sanchez-Lozada*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 month
Contribution to Project:	*Dr. Sanchez Lozada oversaw the administrative aspects of executing the subcontract.*
Funding Support:	*DOD funding*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

For Dr. Richard Johnson, M.D., the following new projects have been initiated:

NIDDK 1RO1DK109408-01A1 (Johnson) 04/01/2016-3/30/2021 2.4 calendar
Dietary Salt has an Unrecognized Role in Modulating $300,000
Energy Intake and Metabolic Syndrome
Goal: To investigate the role of salt in inducing obesity and metabolic syndrome

NIDDK RO1 DK108859-01 (Lanaspa) 04/01/2016-3/30/2021 0.6 calendar
$279,325Targeting fructokinase, endogenous fructose production and purine degradation for the prevention and treatment of hereditary fructose intolerance

Goal: To investigate the role of fructokinase in mice with aldolase B deficiency using aldolase B KO, fructokinase KO and lowering uric acid. This grant has no overlap and does not involve generation of inhibitors.

There have been no changes in active support for Carlos Roncal, Zhilin Song or LG Sanchez Lozada.

What other organizations were involved as partners?

We have a collaboration supported by the DOD with Gaby Sanchez-Lozada at the Cardiologia University as part of our DOD proposal.

Organization Name: Instituto Nacional de Cardiología Ignacio Chavez

Location of Organization: Mexico City, Mexico

Partner's contribution to the project: Will be responsible for completion of aim 3.

In-kind support None

Facilities None

Collaboration None (other than our DOD collaboration with Dr Sanchez-Lozada)

Personnel exchanges None

Other. None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable

9. **APPENDICES:** We are including our paper on desmopressin that is under review at the American Journal of Physiology.

Desmopressin (Type 2) Vasopressin Receptor Agonist Accelerates Heat Stress Nephropathy in Mice

Carlos A. Roncal-Jimenez[1], Tamara Milagres[1], Ana Andres Hernando[1], Masanari Kuwabara[1], Thomas Jensen[1,2], Zhilin Song[1,2], Petter Bjornstad[3], Gabriela E Garcia[1], Yuka Sato[1], Laura G Sanchez-Lozada[4], Miguel A Lanaspa[1], and Richard J Johnson[1]

[1]Division of Renal Diseases and Hypertension, University of Colorado, Aurora, CO, USA; [2]Division of Endocrinology, Metabolism and Diabetes, University of Colorado, Aurora CO; [3]Division of Pediatric Endocrinology, University of Colorado; and [4]Laboratory of Renal Physiopathology, INC Ignacio Chávez, Mexico City, Mexico.

Correspondence to: Richard J Johnson MD, Division of Renal Diseases and Hypertension, Department of Medicine, School of Medicine, University of Colorado, Aurora, Colorado 80045, USA. E-mail: Richard.Johnson@ucdenver.edu. Phone: 303-724-4889.

Supported by a grant from the Department of Defense (W81XWH-14-1-0270). Dr. Kuwabara receives the grant for studying abroad from Federation of National Public Service Personnel Mutual Aid Association in Japan. Drs. Jensen and Bjornstad are also supported by NIH T32 training grants (Dr. Jensen: 5T32DK007446-34, Dr. Bjornstad: T32-DK063687).

Conflicts of Interest: The authors disclose no conflicts of interest related to the manuscript. CRJ, MAL, LGL and RJJ are members of Colorado Research Partners, LLC that is developing inhibitors of fructose metabolism for the treatment of metabolic syndrome and kidney disease. Dr. Johnson is also on the Scientific Board of Amway.

Acknowledgments: This paper is considered a contribution by the University of Colorado Climate Change and Health consortium. We thank Dr Lise Bankir for her suggestions in the experimental design of the study.

Running Title: Desmopressin worsens Heat Stress Nephropathy

Word count Abstract: 222 words; Text words; Tables 1, Figures 5, References 26

Abstract

Background: Recurrent heat stress and dehydration have recently been shown experimentally to cause chronic kidney disease (CKD). One potential mediator may be vasopressin, acting via the type 2 vasopressin receptor (V2 receptor). We tested the hypothesis that desmopressin accelerates CKD in mice subjected to heat stress and recurrent dehydration.

Methods: Recurrent exposure to heat with limited water availability was performed in male mice over a 5 week period, with one group receiving desmopressin twice daily and the other group received vehicle. Two additional control groups were not exposed to heat or dehydration and received vehicle or desmopressin. The effects of the treatment on renal injury was assessed.

Results: Heat stress and recurrent dehydration induced functional changes (albuminuria, elevated urinary NGAL), glomerular changes (mesangiolysis, matrix expansion) and tubulointerstitial changes (fibrosis, inflammation). Desmopressin also induced albuminuria, glomerular changes and tubulointerstitial fibrosis in normal animals, and also exacerbated injury in mice with heat stress nephropathy. Both heat stress and/or desmopressin were also associated with activation of the polyol pathway in the renal cortex, likely due to increased interstitial osmolarity.

Conclusions: Our studies document both glomerular and tubulointerstitial injury and inflammation in heat stress nephropathy, and may be clinically relevant to the pathogenesis of Mesoamerican Nephropathy. Our data also suggest that vasopressin may play a role in the pathogenesis of the renal injury of heat stress nephropathy, likely via a V2-receptor dependent pathway.

Key Words: Vasopressin, Copeptin, Mesoamerican Nephropathy, Chronic Kidney Disease of Unknown Etiology, Heat Stress Nephropathy

Introduction

Historically the development of a pre-renal state was thought to be fully reversible without any permanent kidney damage. However, the identification of an epidemic of chronic kidney disease (CKD) of unknown etiology in Central America has renewed interest in this subject. Specifically, the epidemic is occurring in manual workers on the Pacific coast who are at high risk for recurrent dehydration due to the extreme heat and humidity (8, 12). While initial concerns included toxin exposures, the primary risk factor to date has been recurrent heat stress and dehydration (11, 21, 30). Indeed, the observation of similar epidemics in other countries has led to the suggestion that "heat stress nephropathy" might represent a type of CKD that has not been recognized as a major cause of CKD, but one that might be increasing due to progressive water shortage and climate changes (13).

To better understand the mechanisms by which heat stress might cause CKD, different animal models have been developed in which animals are exposed repetitively to heat and water restriction. Our group has used a model in which animals are exposed multiple times daily to heat stress and dehydration, and over time the animals develop CKD associated with significant scarring of the tubulointerstitium (27). Studies by the Sánchez-Lozada laboratory have used a model of milder heat stress, and while CKD does not occur, one can document marked oxidative stress and renal inflammation (10). In these models hydration with water during the heat stress is protective whereas hydration with sugary beverages makes the injury worse (10, 27).

One of the consequences of heat stress and dehydration is the development of elevated serum osmolarity that activates several mediator systems that may cause renal injury (15). One such system in the polyol-fructokinase pathway. The enzyme aldose reductase is activated by hyperosmolarity (18) and is normally active in the renal medulla where it generates sorbitol from glucose, with the sorbitol acting as an intracellular osmol to protect medullary cells from hypertonicity injury (5). While sorbitol is the endproduct of the polyol pathway in the renal medulla, in the outer medulla and renal cortex the sorbitol can be further degraded to fructose by sorbitol dehydrogenase, and the fructose can then become a substrate for the enzyme fructokinase which is present in the S3 segment of the proximal tubule (6, 23). When fructose is metabolized by fructokinase C, there is a fall in ATP levels, adenine nucleotide turnover, and the production of uric acid, oxidants and chemokines (monocyte chemoattractant protein-1) that can cause local renal injury (6, 23). We recently demonstrated this system is activated with recurrent heat stress and dehydration and that fructokinase knockout mice are protected from the renal damage (27).

The other major mediator system activated by hyperosmolarity is vasopressin. Vasopressin is considered a beneficial hormone as its primary action is to increase water reabsorption in the collecting duct under conditions of hyperosmolarity or extracellular volume depletion. However, there is a 'trade-off' in which chronically elevated vasopressin levels cause renal injury. In fact, most of the supportive data suggest that chronic stimulation of the V2 receptor may lead to kidney damage (1, 7). Specifically the administration of vasopressin, or the V2

agonist desmopressin, induces glomerular hyperfiltration and albuminuria in humans and laboratory animals (2-4).

In this study we sought to examine the role of vasopressin in our heat stress nephropathy model. Because we were worried that blocking vasopressin might result in reduced survival in our water restricted mice, we tested the hypothesis that administration of a V2 agonist would worsen the renal injury in heat stress nephropathy.

Materials and Methods

Experimental Protocol and Animals. Eight week old male C57BL/6J mice (Jackson Labs, Bar Harbor ME) (9) were maintained in temperature- and humidity-controlled specific pathogen-free condition on a 14-hour dark/10-hour light cycle and were fed regular diet *ad libitum* (Harlan Teklad; no. 2918, containing 58 percent carbohydrate, 24 percent protein, and 18 percent fat and devoid of fructose or sugar).

The experimental protocol was as follows. Mice (n=7 per group) were placed in a heat chamber for 30 minutes (39.5 Celsius) each hour over an 8 hour period each week day (Monday to Friday) with water restriction during that time. One group received desmopressin (**Heat + Des**) via subcutaneous injection (dose = 32 ng/Kg dissolved in physiological saline solution (150 ul)) that was administered 30 minutes previous to the initiation of heat stress with a second subcutaneous injection 4 hours later, in the middle of dehydration time). A second group received a subcutaneous injection of physiological saline solution (150 µl) at the same time (**Heat**). Two additional control groups included mice not exposed to heat or dehydration given vehicle (**Control**) or normal mice administered desmopressin (**Des**). This treatment was continued for a 5 week period and resulted in no mortality. Mice were sacrificed at 5 weeks by anesthesia and cardiac exsanguination with collection of serum, urine from bladder, and kidney tissues for the analysis. All experiment were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado.

Biochemical analyses. Urine was collected at the end of the study from the bladder and analyzed for urine osmolarity using Freezing-Point Osmometry (Advance Instruments Micro Osmometer- Norwood Massachusetts USA); urinary albumin concentration was determined by Albuwell M (Exocell, Philadelphia, PA) and urine NGAL was measured using the Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN). Serum creatinine and urine concentration was analyzed with the high-performance liquid chromatography–tandem mass spectrometry method (29). Urinary urea was measured using a Colorimetric QuantiChrom Urea Assay Kit from BioAssay System (Hayward, CA).

Histology. Tissues were fixed in 10% formalin or methyl Carnoy's and embedded in paraffin. Three µm sections were stained with periodic acid-Schiff reagent (PAS). On coronal sections of the kidney, all glomeruli

(50–100 glomeruli) were examined for evaluation of glomerular hypertrophy. Glomerular hypertrophy was determined by outlining the glomerular tuft in all glomeruli and measuring the area using the Aperio software (Aperio Technologies, Vista, CA). Mesangial matrix expansion was determined by measuring the area of type IV collagen deposition in glomeruli using the Aperio software on tissue sections stained for type IV collagen using rabbit anti-type IV collagen antibody (Chemicon International, Temecula, CA) as described elsewhere (20). Specifically, the relative mesangial area (proportion of type IV collagen positive area per glomerular tuft area was calculated using Aperio Software.

Renal fibrosis was determined by staining for type III collagen with a goat anti-type III collagen antibody dilute 1 to 100 (Southern Biotech, Birmingham AL) and digital images at 20X magnification of approximately 10 fields were analyzed using Image scope of Aperio Scanner software. The percent positive area was determined as the 3,3-diaminobenzidine-positive pixel values per negative pixel values in each section. Infiltrating macrophages was detected using the mouse F4/80 monoclonal antibody (Serotec, Oxford, UK) respectively. The number of positive cells for F4/80 was counted using an Aperio scanner (Aperio Technologies, Vista, CA). The software allows color recognition and positive cells were identified as % positive color saturation at 20 magnification in a blinded manner using at least 15 fields for each biopsy sample.

Western blotting and Renal MCP-1 measurement. Renal cortical MCP-1 levels were measured in kidney tissue homogenates using the MCP-1 Elisa Kit Invitrogen (Life Technologies). Briefly, tissues (~50 mg) were homogenized in 500 µl of mitogen-activated protein kinase lysis buffer (19) containing 0.5% triton X-100, 2 mM $MgCl_2$, 1 mM EGTA and 1 mM dithiothreitol supplemented with protease and phosphatase inhibitors (Roche). Samples were then incubated on ice for 30 min with occasional vortex and spun at 13,000 r.p.m. for 15 min at 4 ^{o}C and the assay for MCP-1 performed with correction for total protein using the BCA protein assay Kit (Pierce, Rockford, IL).

For Western blotting, kidney lysates using the same mitogen-activated protein kinase lysis buffer (19) were prepared from mouse cortical tissue (~50 mg) and were incubated on ice for 30 min with occasional vortex and then spun at 13,000 r.p.m. for 15 min at 4 ^{o}C. The supernatant was collected and content determined by the BCA protein assay (Pierce, Rockford, IL), and then 50 µg protein was loaded per lane for SDS–PAGE (10% w/v) analysis and transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies (all of them at a 1:1,000 dilution; (Fructokinase (Sigma, HPA007040); Aldose reductase (rabbit anti-mouse aldose reductase (AKR1B1) antibody (Novus Biologicals, Littleton, CO)) β-Actin, Cell Signaling 4967S; KHK, Sigma HPA007040) and were visualized using a horseradish peroxidase secondary antibody(1:2,000) and the HRP Supersignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). Chemiluminescence was recorded with an Image Station 440CF and results were analyzed with the 1D Image Software (Kodak Digital Science, Rochester, NY).

Kidney homogenized supernatant cortex was collected for uric acid measurement using QuantiChrom Uric Acid assay kit by BioAssay Systems (CA) .

Statistical analysis. Statistical analysis was performed using one-way analysis Bonferroni of variance by rank. A two-sided value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the GraphPad Prism version 6 (GraphPad Software, Inc. La Jolla, CA). All data are presented as the mean ± s.e.m.

Results

Hydration Status and Effect of Desmopressin. The model of heat stress nephropathy we used is one in which animals are recurrently exposed to heat over an 8 hour period with complete water restriction during that time (27). It results in relatively severe acute dehydration, with approximately 14% weight loss at the end of the heating period, but animals fully rehydrate at night and there is no mortality and body weights remain stable over the 5 week period (Table 1) (27). However, by the end of the dehydration/heat stress period, there is a marked increase in serum osmolality that occurs in association with an increase in serum copeptin (a more stable peptide derived from the same precursor molecule as vasopressin (17)) and a rise in urinary osmolality and urine creatinine, consistent with urinary concentration (**Figure 1**). The administration of desmopressin to animals exposed to similar levels of heat stress and dehydration was also associated with similar findings as with heat stress alone, although the rise in serum osmolality was blunted likely from water reabsorption in the collecting duct due to the action of desmopressin (Figure 1). In contrast, when desmopressin was administered to healthy, hydrated rats, the serum osmolality tended to decrease and the urine became concentrated as noted by the rise in the urine osmolality and urinary creatinine (**Figure 1**). Compared to those animals subjected to heat stress, the urine osmolality tended to be slightly higher in the healthy mice administered with desmopressin, which might reflect the fact that these former animals were developing chronic tubulointerstitial disease that might partially impair their urinary concentration ability (see later).

Effect of Heat Stress and Desmopressin on Renal Function. Serum creatinine (measured by HPLC) was no different among groups. Serum BUN levels tended to be elevated in mice receiving desmopressin with or without heat stress, but this was not significant. However, albuminuria increased stepwise with desmopressin alone, followed by heat stress, and then by heat stress plus desmopressin (**Figure 1**). Urine NGAL was also significantly elevated in both desmopressin groups and to a lesser extent in the heat stress alone group compared to normal healthy controls (**Figure 1**). Whether this reflects renal injury or a pre-renal state remains unclear, because urinary NGAL has also been reported to be elevated in pre-renal conditions (24).

Effect of Heat Stress and Desmopressin on Renal Histology. Light microscopy (PAS staining) was qualitatively associated with a stepwise increase in proximal tubular injury (with loss of brush border) with desmopressin, followed by heat stress/dehydration, and then combined desmopressin and heat stress/dehydration (**Figure 2**). Similar findings were shown for interstitial inflammation (macrophage infiltration) and for interstitial collagen deposition (**Figure 2 and 3**). The stepwise increase in inflammation was also associated with a stepwise increase in serum monocyte chemoattractant protein-1 (MCP-1) and in renal cortical MCP-1 levels.

There were also glomerular changes observed, with both mesangiolysis and mesangial deposition of collagen IV that were significant in animals treated with desmopressin alone, heat alone or the combination (**Figure 4**).

Effect of Desmopressin and Heat on the Aldose Reductase-Fructokinase Pathway. Evidence for increased activity of the polyol-fructokinase pathway with heat stress was suggested by the significant increase in both cortical aldose reductase protein and uric acid concentrations and a tendency for higher fructose, fructokinase and sorbitol levels. Treatment with desmopressin in normal mice also led to higher aldose reductase, fructose and uric acid levels. Similarly, the combination of desmopressin with heat stress also resulted in higher fructose and uric acid levels compared to control mice (**Figure 5**).

Vasopressin is known to increase the reabsorption of urea, which helps in urinary concentration (33). An increase in interstitial urea might extend into the renal cortex where it might facilitate water absorption by the cortical collecting duct. Consistent with this finding, we found that desmopressin increased cortical urea concentration in normal mice (Figure 5). These effects were not observed in mice with heat-induced injury.

Discussion

In this study, we explored the role of vasopressin, and particularly the V2 receptor, in a model of heat stress nephropathy. Our hypothesis was that stimulation of the V2 receptor would accelerate the renal injury associated with heat stress nephropathy. Our primary finding was that the administration of desmopressin, a V2 agonist, markedly accelerated renal injury with greater glomerular and tubulointerestitial damage. The most deleterious effect of desmopressin appeared to be on the glomerulus (mesangiolysis and collagen IV deposition), because the addition of desmopressin resulted in significantly worse glomerular injury compared to heat stress alone. We also found evidence that stimulation of the V2 receptor could activate the polyol-fructokinase pathway independent of heat stress-induced injury, as noted by its effects on renal fructose and uric acid accumulation. These data therefore suggest that vasopressin is likely a mediator of renal injury under conditions of recurrent heat stress and dehydration, and again emphasize the importance of maintaining good hydration and normal serum osmolarity to promote renal health.

Desmopressin, and vasopressin, have previously been reported to cause glomerular hyperfiltration and albuminuria in both experimental animals and in humans (2-4). Since V2 receptors are not present in glomeruli, the mechanism for how vasopressin induces these changes are not completely understood, although it has been posited to be by altering tubuloglomerular feedback (4). Alternatively, studies by Bonventre et al. have shown that isolated injury to the proximal tubule may also cause glomerular injury (14). Whether this may represent a similar process is unknown.

One potential explanation could be cross-talk between the vasopressin and fructokinase systems. Thus, vasopressin, by increasing urea and sodium reabsorption, will increase interstitial osmolarity. Aldose reductase has an osmolarity-sensitive promoter, and activity increases in parallel with increasing osmolarity (28). We observed a significant increment in cortical urea with desmopressin, however this effect was not significant in mice exposed to both heat and desmopressin (**Figure 5**). Nevertheless, renal cortical fructose and uric acid were elevated both with desmopressin alone and with heat stress and desmopressin. This apparent paradox might be explained by the tonic effect exerted by other organic osmolytes, such as betaine, inositol, taurine, and glycerophosphocholine in the context of heat stress. Changes in aldose reductase and fructokinase protein were more variable, but protein concentrations do not always correlate with activity. One potential reason for the variable findings may relate to the development of renal injury, which might alter the ability of the renal medullary tubules to maintain high interstitial osmolarity. This could explain why cortical urea was not as high in mice with heat-associated injury compared to desmopressin alone.

The generation of fructose in the renal cortex is associated with fructokinase-dependent tubular injury both in response to heat stress and in diabetes (20, 27). Fructose induces tubular injury due to the generation of uric acid, oxidative stress, and the production of chemokines such as MCP-1 (20, 26, 27). In turn, we have shown in a model of diabetic nephropathy that fructokinase-mediated tubular injury is also associated with worse albuminuria and glomerular injury (20), suggesting this might represent the link between vasopressin and glomerular injury. Consistent with this cross-talk, we have recently reported evidence for tubular injury in patients with chronic hyponatremia, and in these patients urinary fructose levels are elevated (22) In addition, fructose also stimulates vasopressin release (10, 32), thereby leading to a positive feedback system (16).

The relevance of these findings to the epidemic of CKD in Mesoamerica and elsewhere is evident. An interesting finding from this study is that both glomerular and tubulointerstitial injuries were identified in our heat stress model. We had previously focused on the tubulointerstitial injuries as they were most evident, but in this model the addition of desmopressin also led to significant glomerular injury. In this regard, glomerular injury and glomerulosclerosis have been observed in Mesoamerican Nephropathy, and have been proposed to not be simply secondary to the tubulointerstitial injury but to represent a primary process (31). Thus, this model may be especially relevant to this observation.

In summary, these studies provide evidence that both the aldose reductase-fructokinase system (27) and vasopressin play causal roles in heat stress nephropathy. Rehydration with sugary beverages also accelerates this pathway not only by providing fructose substrate but also by stimulating vasopressin (10). Our studies also suggest uric acid may be involved, due to its known ability to stimulate inflammatory pathways, and also because of its potential crystal-dependent and independent effects (25, 26). Thus, we encourage further studies to investigate whether these processes may be involved in the current epidemics of CKD occurring under conditions of documented heat stress (13).

Table 1 General Characteristics of Experimental Groups.

	Control (n=7)	Desmopressin (n=7)	Heat (n = 7)	Heat+ Desmo (n = 7)	P values
Body weight Basal (g)	22.4 ± 1.4	23.5 ± 1.2	23.5 ± 1.1	23.5 ± 1.8	NS
Body weight After 5 W (g)	24.2 ± 1.2	24.4 ± 1.7	23.0 ± 0.6	23.0 ± 1.4	NS
(%) Body weight Loss after Dehydration			14.1 ± 1.1	14.5 ± 3.0	NS

Key: Desmo, desmopressin

Figure 1. **Effects of Desmopressin on Hydration Status and Renal Function**. Serum osmolality (Figure A) increased in the heat stress control group and tended to be lower in the heat group that received desmopressin. Urine osmolality (Figure B) and urine creatinine (Figure C) increased with heat stress but also in both groups receiving desmopressin. Serum copeptin (Figure D) increased in the two groups of mice exposed to heat stress and dehydration. No differences in serum creatinine (Figure E) was observed; whereas an increase in serum BUN (blood urea nitrogen) (Figure F) tended to increase in the desmopressin groups. Urine albumin (Figure G) stepwise increased with desmopressin alone, followed by heat stress/dehydration, and then heat stress plus desmopressin. Urinary NGAL excretion (Figure H) was highest in groups receiving desmopressin. *p<0.05; **p<0.01; ***p<0.001

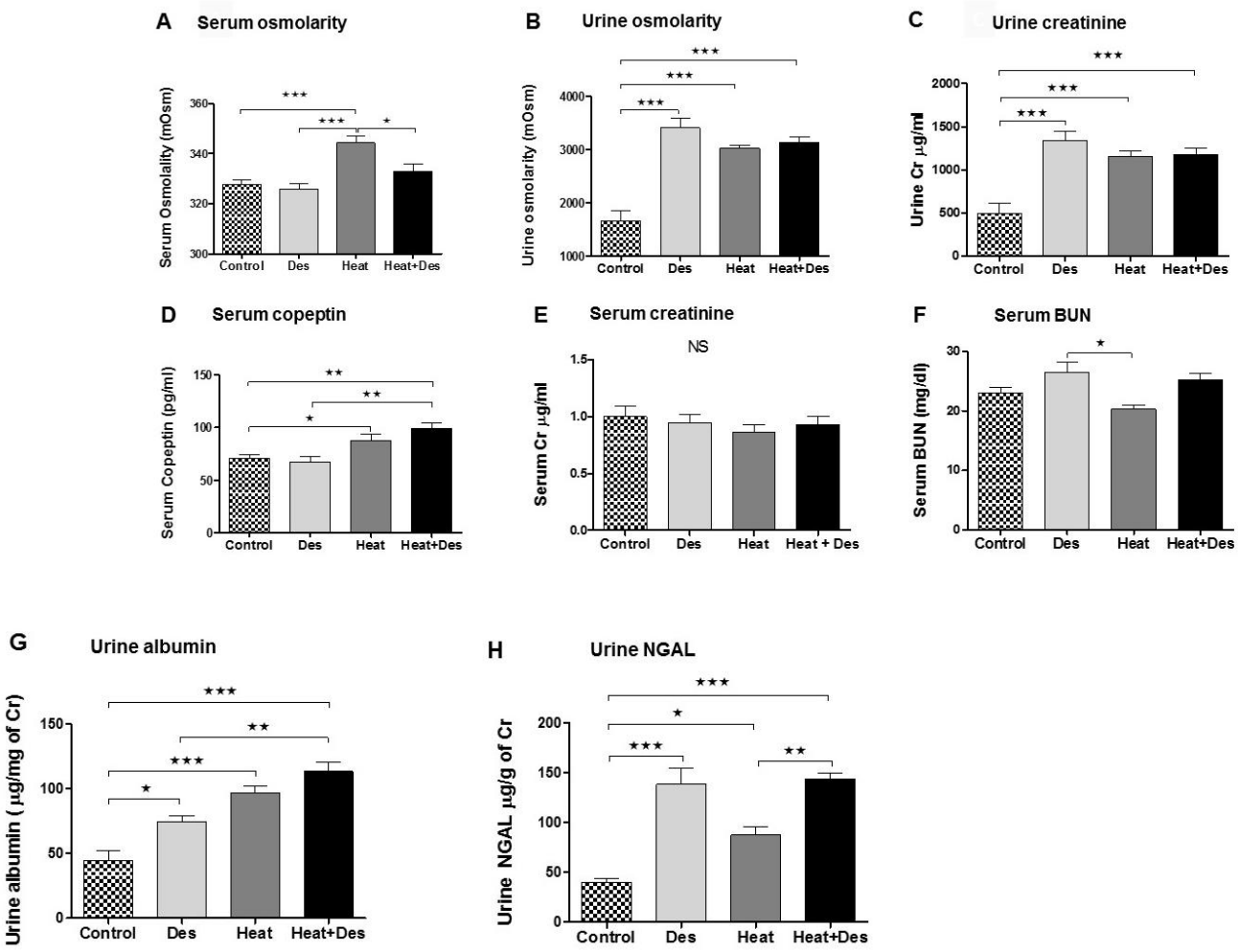


Figure 2. Histological Changes: Qualitative Changes. Proximal tubular injury (loss of brush border with tubular dilation- showed in black arrows) was observed in Des; Heat; and Heat+Des groups (Fig. PAS: B, C, D). Macrophage infiltration (F4/80; Fig. E, F, G, H) was increased in Des; Heat and Heat+ Des groups compared with the Control group (see black arrows). Interstitial collagen III deposition was minimal in normal controls (Figure I), but was increased in the Des; Heat and Heat + Des groups (Fig. J, K, L).

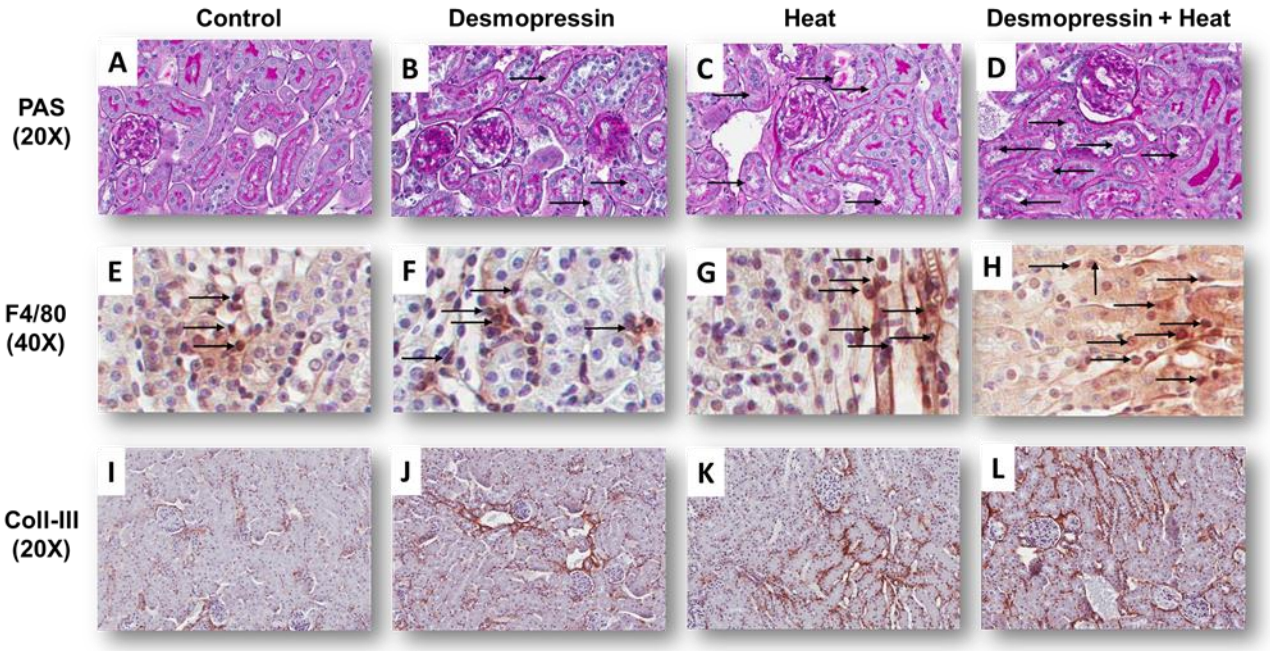


Figure 3. Histologic Injury: Semi-quantitative Changes: Serum MCP-1 represent a biomarker of inflammation and was increased in the animals exposed to heat and tended to worsen with desmopressin (Figure A). Renal cortical MCP-1 protein levels were also elevated in the heat stress plus desmopressin group with a tendency to increase in the heat group (Fig. B). Consistent with increased levels of the MCP-1 chemokine, infiltrating macrophages (Figure C, noted by F4/80 scoring) were significantly elevated with heat stress and was worse in the heat stress group receiving desmopressin. Interstitial collagen III deposition paralleled the inflammatory changes (Fig. D). Statistical analysis used Bonferroni one-way analysis of variance by ranks. A two-sided value of P <0.05 was considered statistically significant. *p<0.05; **p<0.01; ***p<0.001

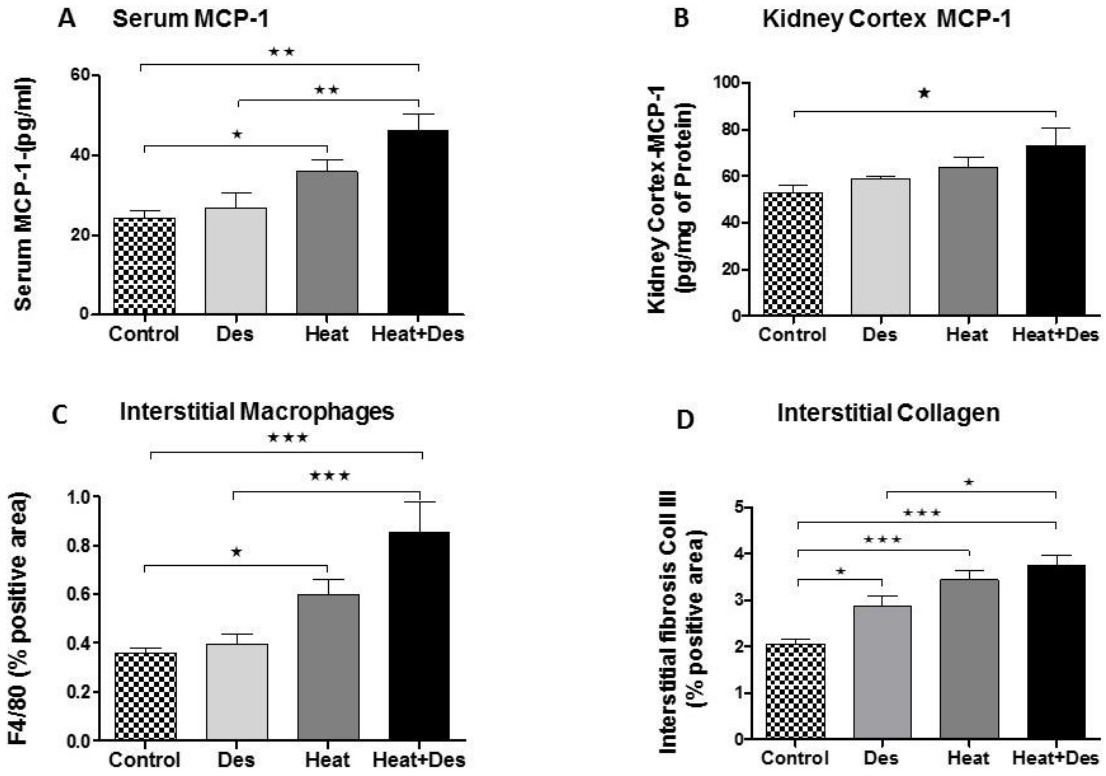


Figure 4. Focal glomerular mesangiolysis. Heat stress was associated with focal mesangiolysis as were control mice administered desmopressin compared to healthy control mice (Figure A-D, Quantification of mean percent glomeruli involved in Figure I). This was accompanied by parallel changes in mesangial matrix expansion (as noted by type IV collagen immunostaining, Fig. E-H), with quantification in Fig. J. The effects of desmopressin to induce mesangiolysis and mesangial expansion of type IV collagen were significant. (Des=Desmopressin)**.** *p<0.05; **p<0.01; ***p<0.001

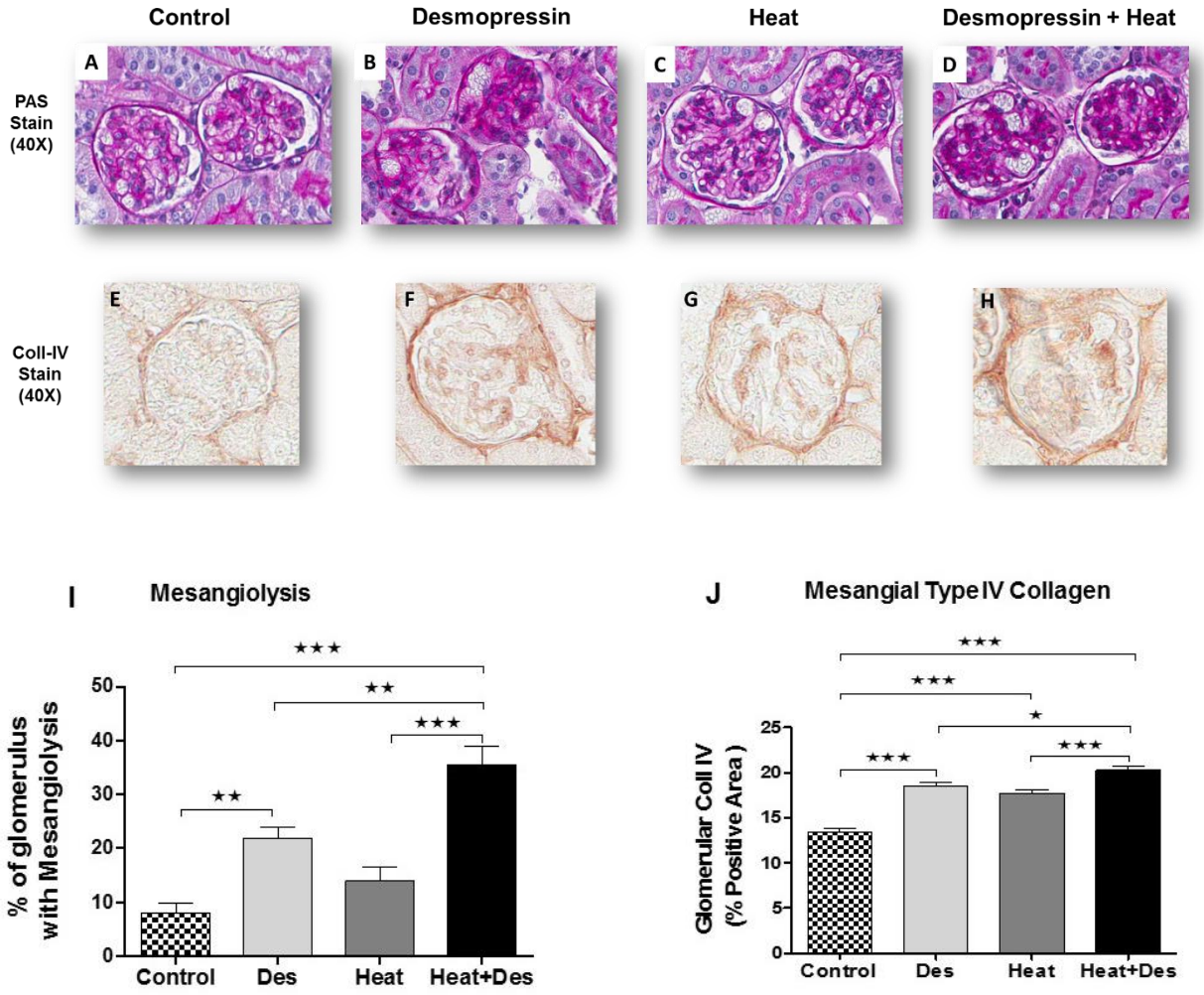
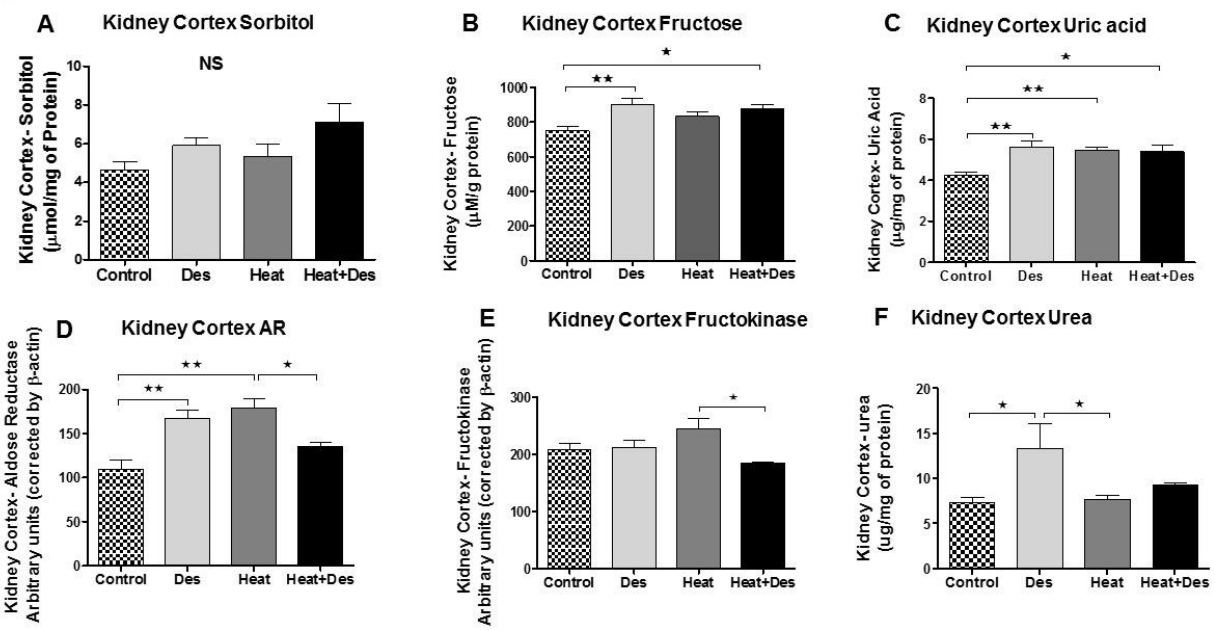


Figure 5. Effect of Desmopressin on the Polyol-Fructokinase Pathway in the Kidney Cortex. Renal cortical homogenates tended to show higher levels of sorbitol (Figure A) with heat stress and/or desmopressin but this was not significant. In contrast, renal cortical fructose (Figure B) and uric acid (Figure C) were higher with desmopressin independently of whether animals were exposed to heat stress, and uric acid was also elevated in animals with heat stress alone compared to healthy controls. Western blot documented increase aldose reductase protein in renal cortex of desmopressin controls and mice exposed to heat, with a tendency for higher expression in animals exposed to heat and desmopressin (Figure D). Fructokinase protein tended to be higher in the heat exposed mice compared to control but this was not significant (Figure E). Cortical urea concentration (Figure F) was observed in control animals receiving desmopressin. *p<0.05; **p<0.01; ***p<0.001



References

5. 1. **Bankir L, Bouby N, and Ritz E.** Vasopressin: a novel target for the prevention and retardation of kidney disease? *Nat Rev Nephrol* 9: 223-239, 2013.
6. 2. **Bardoux P, Bichet DG, Martin H, Gallois Y, Marre M, Arthus MF, Lonergan M, Ruel N, Bouby N, and Bankir L.** Vasopressin increases urinary albumin excretion in rats and humans: involvement of V2 receptors and the renin-angiotensin system. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 18: 497-506, 2003.
7. 3. **Bardoux P, Martin H, Ahloulay M, Schmitt F, Bouby N, Trinh-Trang-Tan MM, and Bankir L.** Vasopressin contributes to hyperfiltration, albuminuria, and renal hypertrophy in diabetes mellitus: study in vasopressin-deficient Brattleboro rats. *Proceedings of the National Academy of Sciences of the United States of America* 96: 10397-10402, 1999.
8. 4. **Bouby N, Ahloulay M, Nsegbe E, Dechaux M, Schmitt F, and Bankir L.** Vasopressin increases glomerular filtration rate in conscious rats through its antidiuretic action. *Journal of the American Society of Nephrology : JASN* 7: 842-851, 1996.
9. 5. **Burg MB.** Molecular basis of osmotic regulation. *The American journal of physiology* 268: F983-996, 1995.
10. 6. **Cirillo P, Gersch MS, Mu W, Scherer PM, Kim KM, Gesualdo L, Henderson GN, Johnson RJ, and Sautin YY.** Ketohexokinase-dependent metabolism of fructose induces proinflammatory mediators in proximal tubular cells. *Journal of the American Society of Nephrology : JASN* 20: 545-553, 2009.
11. 7. **Clark WF, Sontrop JM, Huang SH, Moist L, Bouby N, and Bankir L.** Hydration and Chronic Kidney Disease Progression: A Critical Review of the Evidence. *American journal of nephrology* 43: 281-292, 2016.
12. 8. **Correa-Rotter R, Wesseling C, and Johnson RJ.** CKD of Unknown Origin in Central America: The Case for a Mesoamerican Nephropathy. *American journal of kidney diseases : the official journal of the National Kidney Foundation* 63: 506-520, 2014.
13. 9. **Diggle CP, Shires M, McRae C, Crellin D, Fisher J, Carr IM, Markham AF, Hayward BE, Asipu A, and Bonthron DT.** Both isoforms of ketohexokinase are dispensable for normal growth and development. *Physiol Genomics* 42A: 235-243, 2010.
14. 10. **García-Arroyo FE, Cristobal M, Arellano-Buendia AS, Osorio H, Tapia E, Soto V, Madero M, Lanaspa M, Roncal-Jimenez CA, Bankir L, Johnson RJ, and Sanchez-Lozada LG.** Rehydration with Soft Drink-like Beverages Exacerbates Dehydration and Worsens Dehydration-associated Renal Injury *American journal of physiology Regulatory, integrative and comparative physiology* in press: 2016.
15. 11. **Garcia-Trabanino R, Jarquin E, Wesseling C, Johnson RJ, Gonzalez-Quiroz M, Weiss I, Glaser J, Jose Vindell J, Stockfelt L, Roncal C, Harra T, and Barregard L.** Heat stress, dehydration, and kidney function in sugarcane cutters in El Salvador--A cross-shift study of workers at risk of Mesoamerican nephropathy. *Environmental research* 142: 746-755, 2015.
16. 12. **Garcia Trabiano R.** Nefropatía terminal en pacientes de un

hospital de referencia en El Salvador. *Rev Panam Salud Publica/Pan Am J Public Health* 12: 2002.
17. 13. **Glaser J, Lemery J, Rajagopalan B, Diaz HF, Garcia-Trabanino R., Taduri G, Madero M, Amarasinghe MD, Abraham G, Anutrakulchai S, Jha V, Stenvinkel P, Roncal-Jiménez C, Lanaspa M, Correa-Rotter R, Sheik-Hamad D, Burdmann EA, Andres-Hernando A, MIlagres T, Weiss I, Kanbay M, Wesseling C, Sanchez-Lozada LG, and Johnson RJ.** Climate Change and the Emergent Epidemic of Chronic Kidney Disease from Heat Stress in Rural Communities: The Case for Heat Stress Nephropathy. *CJASN* in press: 2016.
18. 14. **Grgic I, Campanholle G, Bijol V, Wang C, Sabbisetti VS, Ichimura T, Humphreys BD, and Bonventre JV.** Targeted proximal tubule injury triggers interstitial fibrosis and glomerulosclerosis. *Kidney international* 82: 172-183, 2012.
19. 15. **Johnson RJ, Rodriguez-Iturbe B, Roncal-Jimenez C, Lanaspa MA, Ishimoto T, Nakagawa T, Correa-Rotter R, Wesseling C, Bankir L, and Sanchez-Lozada LG.** Hyperosmolarity drives hypertension and CKD--water and salt revisited. *Nat Rev Nephrol* 10: 415-420, 2014.
20. 16. **Johnson RJ, Stenvinkel P, Jensen T, Lanaspa M, Roncal C, Song Z, Bankir L, and Sanchez-Lozada LG.** Metabolic and kidney diseases in the setting of climate change, water shortage, and survival factors. *JASN* in press: 2016.
21. 17. **Khan SQ, Dhillon OS, O'Brien RJ, Struck J, Quinn PA, Morgenthaler NG, Squire IB, Davies JE, Bergmann A, and Ng LL.** C-terminal provasopressin (copeptin) as a novel and prognostic marker in acute myocardial infarction: Leicester Acute Myocardial Infarction Peptide (LAMP) study. *Circulation* 115: 2103-2110, 2007.
22. 18. **Ko BC, Ruepp B, Bohren KM, Gabbay KH, and Chung SS.** Identification and characterization of multiple osmotic response sequences in the human aldose reductase gene. *The Journal of biological chemistry* 272: 16431-16437, 1997.
23. 19. **Lanaspa MA, Almeida NE, Andres-Hernando A, Rivard CJ, Capasso JM, and Berl T.** The tight junction protein, MUPP1, is up-regulated by hypertonicity and is important in the osmotic stress response in kidney cells. *Proceedings of the National Academy of Sciences of the United States of America* 104: 13672-13677, 2007.
24. 20. **Lanaspa MA, Ishimoto T, Cicerchi C, Tamura Y, Roncal-Jimenez CA, Chen W, Tanabe K, Andres-Hernando A, Orlicky DJ, Finol E, Inaba S, Li N, Rivard CJ, Kosugi T, Sanchez-Lozada LG, Petrash JM, Sautin YY, Ejaz AA, Kitagawa W, Garcia GE, Bonthron DT, Asipu A, Diggle CP, Rodriguez-Iturbe B, Nakagawa T, and Johnson RJ.** Endogenous fructose production and fructokinase activation mediate renal injury in diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* 25: 2526-2538, 2014.
25. 21. **Laws RL, Brooks DR, Amador JJ, Weiner DE, Kaufman JS, Ramirez-Rubio O, Riefkohl A, Scammell MK, Lopez-Pilarte D, Sanchez JM, Parikh CR, and McClean MD.** Changes in kidney function among Nicaraguan sugarcane workers. *International journal of occupational and environmental health* 21: 241-250, 2015.
26. 22. **Lee S-MK, Lanaspa MA, Sánchez-Lozada LG, and Johnson RJ.** Hyponatremia with Persistent Elevated Urinary Fractional Uric Acid Excretion: Evidence for Proximal Tubular Injury? *Kidney & blood pressure research* in press: 2016.

27. 23. **Nakayama T, Kosugi T, Gersch M, Connor T, Sanchez-Lozada LG, Lanaspa MA, Roncal C, Perez-Pozo SE, Johnson RJ, and Nakagawa T.** Dietary fructose causes tubulointerstitial injury in the normal rat kidney. *American journal of physiology Renal physiology* 298: F712-720, 2010.
28. 24. **Nejat M, Pickering JW, Devarajan P, Bonventre JV, Edelstein CL, Walker RJ, and Endre ZH.** Some biomarkers of acute kidney injury are increased in pre-renal acute injury. *Kidney international* 81: 1254-1262, 2012.
29. 25. **Roncal-Jimenez C, Garcia-Trabanino R, Barregard L, Lanaspa MA, Wesseling C, Harra T, Aragon A, Grases F, Jarquin ER, Gonzalez MA, Weiss I, Glaser J, Sanchez-Lozada LG, and Johnson RJ.** Heat Stress Nephropathy From Exercise-Induced Uric Acid Crystalluria: A Perspective on Mesoamerican Nephropathy. *American journal of kidney diseases : the official journal of the National Kidney Foundation* 67: 20-30, 2016.
30. 26. **Roncal CA, Mu W, Croker B, Reungjui S, Ouyang X, Tabah-Fisch I, Johnson RJ, and Ejaz AA.** Effect of elevated serum uric acid on cisplatin-induced acute renal failure. *American journal of physiology Renal physiology* 292: F116-122, 2007.
31. 27. **Roncal Jimenez CA, Ishimoto T, Lanaspa MA, Rivard CJ, Nakagawa T, Ejaz AA, Cicerchi C, Inaba S, Le M, Miyazaki M, Glaser J, Correa-Rotter R, Gonzalez MA, Aragon A, Wesseling C, Sanchez-Lozada LG, and Johnson RJ.** Fructokinase activity mediates dehydration-induced renal injury. *Kidney international* 86: 294-302, 2014.
32. 28. **Ruepp B, Bohren KM, and Gabbay KH.** Characterization of the osmotic response element of the human aldose reductase gene promoter. *Proceedings of the National Academy of Sciences of the United States of America* 93: 8624-8629, 1996.
33. 29. **Takahashi N, Boysen G, Li F, Li Y, and Swenberg JA.** Tandem mass spectrometry measurements of creatinine in mouse plasma and urine for determining glomerular filtration rate. *Kidney international* 71: 266-271, 2007.
34. 30. **Wesseling C, Aragon A, Gonzalez M, Weiss I, Glaser J, Bobadilla NA, Roncal-Jimenez C, Correa-Rotter R, Johnson RJ, and Barregard L.** Kidney function in sugarcane cutters in Nicaragua - A longitudinal study of workers at risk of Mesoamerican nephropathy. *Environmental research* 147: 125-132, 2016.
35. 31. **Wijkstrom J, Leiva R, Elinder CG, Leiva S, Trujillo Z, Trujillo L, Soderberg M, Hultenby K, and Wernerson A.** Clinical and pathological characterization of mesoamerican nephropathy: a new kidney disease in central america. *American journal of kidney diseases : the official journal of the National Kidney Foundation* 62: 908-918, 2013.
36. 32. **Wolf JP, Nguyen NU, Dumoulin G, and Berthelay S.** Influence of hypertonic monosaccharide infusions on the release of plasma arginine vasopressin in normal humans. *Horm Metab Res* 24: 379-383, 1992.
37. 33. **Yang B, and Bankir L.** Urea and urine concentrating ability: new insights from studies in mice. *American journal of physiology Renal physiology* 288: F881-896, 2005.